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Linezolid as an eradication agent against assembled methicillin-resistant *Staphylococcus aureus* biofilms†

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Methicillin-resistant *Staphylococcus aureus* (MRSA) infections are a major health problem worldwide. One of the therapeutic options for treating MRSA is linezolid (LZD), which acts by binding to the ribosome bacteria and inhibiting protein synthesis. Bacterial biofilms are assembled communities which are around 10 to 1000 times more resistant to antibiotics than their planktonic counterparts. The aim of this work was to investigate the inhibition profile and the percentage of biofilm eradication in clinical and reference *S. aureus* strains caused by LZD. The bacterial biomass was assessed by crystal violet staining, and biofilm formation was studied using the XTT assay, with mature biofilm samples being exposed to the antibiotic and the inhibition profile also being measured by XTT. Antibiofilm activity was studied at different times by SEM to evaluate LZD eradication. All the tested strains produced a biofilm matrix, with clinical MRSA forming more biomass. The antibiofilm activity was observed at 10× MIC and revealed that LZD eliminated at least 98% of cell bunch clusters. Our results suggest that LZD is an efficient agent for eradicating MRSA biofilms.

Introduction

Hospital-acquired methicillin-resistant *Staphylococcus aureus* (MRSA) occurs worldwide, with community strains of MRSA continuing to proliferate. Alarming data provided by the World Health Organization indicates that patients infected with MRSA have a mortality probability 64% higher than those infected by non-resistant strains.[1]

As biofilms are involved in up to 80% [2] of bacterial infections, there is an urgently increasing need to prevent their formation because they can lead to greater antibiotic resistance.[3,4] These organized communities secrete an extracellular matrix composed of exopolymeric substances (EPS),[4] with the matrix playing an efficient role in reducing the physical interaction between antimicrobials and bacteria, and consequently, increasing the number of treatment failures.

The activity of numerous antimicrobials, such as linezolid (LZD), vancomycin, daptomycin, has been investigated against assembled *S. aureus* biofilms. Many of these evaluations have produced contradictory findings,[5] as result of the applied methodology, tested strains and culture growth conditions, among other factors. For the present study, we selected the commercially available oxazolidinone, LZD, because it is one of the few therapeutic options to fight against MRSA infections. LZD has been marketed for the past 15 years, and other analogues are also currently under investigation.[6] It functions by binding to the 50S subunit of the prokaryotic ribosome, thereby preventing it from complexing with the 30S subunit, mRNA, initiation factors and formylmethionyl-tRNA.[7,8] Mendes *et al.* (2014)[9] reported on a study performed in the USA between 2004 and 2012, in which the percentage of LZD-resistance strains was around 0.2%. The study included 2980 *S. aureus* isolates; 1537 oxacillin-susceptible and 1443 oxacillin-resistant, all were inhibited by linezolid at 2 μg mL^{-1}, except for three isolates displaying MIC values at 4 and 32 μg mL^{-1}, respectively. Also a surveillance program driven through the same period on 25 000 *S. aureus* isolates the percentages of resistance rates to the oxazolidinone were under 0.1% considering countries from all continents.

In order to contribute to the studies published on LZD and its antibiofilm ability, we conducted our research on clinical methicillin-resistant *S. aureus* strains and also sensitive ones to evaluate antibiotic activity against biofilms.

Experimental

Chemical and reagents

Linezolid, XTT ({2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide}), phenazine

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methosulphate (PMS) and glucose were purchased from Sigma®. Solvents were of analytical reagent grade were acquired from Cicarelli (Argentina). Crystal violet and agar media were purchased from Britania (Argentina).

Bacterial strains and growth conditions

The *S. aureus* strains employed in this study were *S. aureus* ATCC 29213 (MSSA ATCC 29213), methicillin-resistant *S. aureus* ATCC 43300 (MRSA ATCC 43300), four methicillin-resistant clinical isolates of *S. aureus* (MRSA 1, MRSA 2, MRSA 3, MRSA 4) and a susceptible clinical strain (MSSA 5). Clinical isolates were supplied by Sanatorio Aconcagua, Córdoba, Argentina.

Stock cultures were preserved at −70 °C using glycerol 10% (v/v) as a cryoprotectant. The strains were grown in tryptic soy agar (TSA) at 37 °C for 18 h, after which, bacterial culture was prepared by inoculating one single colony from a pure culture in tryptic soy broth (TSB).

To produce a strong biofilm formation, bacteria were grown in TSB supplemented with 0.25% glucose. After overnight incubation under uninterrupted shaking at 130 rpm, the well-plate was rinsed with sterile buffer saline PBS to remove planktonic bacteria, before adding, fresh TSB media to the attached biofilm to carry out the experiments.[10]

Minimum inhibitory concentration (MIC)

MIC was assessed by the macro-broth dilution method following CLSI guidelines.[11] Overnight cultures of each strain were prepared in Mueller Hinton Broth (MHB) and diluted to 10^6 colony forming units (CFU) mL^{-1}. MIC determination was carried out for 2-fold diluted solutions of linezolid (LZD) prepared in phosphate saline buffer (PBS) pH 7, at concentrations ranging from 8 μg mL^{-1} to 0.015 μg mL^{-1}. The lowest antimicrobial concentration at which growth was completely inhibited after overnight incubation of the tubes at 37 °C was the MIC. Bacteria controls, without antimicrobial, were run in parallel.

Efficacy of linezolid on self-assembled biofilm

The *S. aureus* mature biofilms were formed in a 96 well-plate to evaluate the efficacy of LZD on biofilms. For these self-assembled biofilms, the supernatant was discarded and the well-plate rinsed three times with PBS. Subsequently, medium containing antibiotic (10 to 200 μg mL^{-1}) was added and the sample was incubated for 24 h at 37 °C under continuous shaking, after which, the biomass formation was assessed by crystal violet staining (CV). First, the biofilm was washed with PBS, before being fixed with methanol for 15 minutes, rinsed and dumped. Finally, CV was added to stain the matrix for 5 minutes, which was dried at room temperature, and the well-plate was measured at 595 nm using a spectrophotometer.[12]

Bacterial viability was measured using 2,3-(2-methoxy-4-nitro-5-sulphophenyl)-5-[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide (XTT) at 490 nm.[13] The biofilms treated with LZD or control (untreated) were washed three times with PBS, and solutions of XTT and PMS (phenazine methosulfate) were prepared at a final concentration of 200 μg mL^{-1} and 20 μg mL^{-1}, respectively. After mixing these two solutions, an aliquot of 250 μL was added to each well and incubated in the dark for 3 h at 37 °C.

Biofilm eradication and structure

Mature biofilm samples were formed over a coverslip and treated with LZD (10× MIC) as described above, after which, samples were rinsed three times with PBS. The disks were dehydrated with increasing ethanol percentages (50, 70, 80, 90 and 100%), and each disk was gold sputter-coated and mounted on a cover glass.[14] Images were captured by SEM using a Carl Zeiss Σigma scanning electron microscope at the Laboratorio de Microscopía y Análisis por Rayos X (LAMARX) of Universidad Nacional de Córdoba, and further processed using the Fiji-ImageJ software, which is in the public domain.[15] Images were randomly taken and eight were carefully chosen to set the image scale (μm) and measure the biofilm area on the glass (2769 μm^2). In addition, a median filter and thresholding (Otsu) were used and binary images were obtained automatically.[16] Finally, biofilm area on the cover was measured to obtain the biofilm eradication percentage.

Results and discussion

Biofilm biomass quantification

All the strains used in this study were susceptible to LZD, with the MICs of planktonic strains being 2 μg mL^{-1} for MRSA 1, MRSA 2, MRSA 3, MSSA 5, and MSSA ATCC 29213 and 1 μg mL^{-1} for MRSA ATCC 43300 and MRSA 4.

The biomass assay revealed a higher biofilm production in the MRSA 2, MRSA ATCC 43300 and MSSA ATCC 29213 strains than MRSA 3, MRSA 4 or MSSA 5, with the strain that produced the highest biomass being the MRSA 1 (Fig. 1). The biomass average value was determined by four replicates performed at least four times on independent days, and interpreted using the following scale: high (>0.24), low (>0.12 and <0.24) or negative (<0.12). Here, all the clinical and reference strains studied presented a high value in agreement with Baldassarri *et al.* (1993).[17] Curiously, the methicillin-resistant strains exhibited

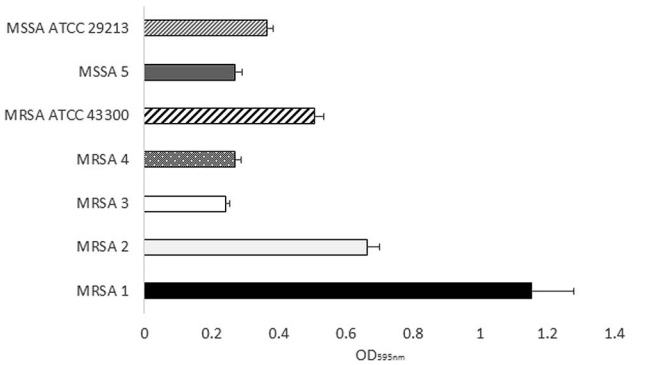

Fig. 1 Biofilm production of *Staphylococcus aureus* clinical and reference strains, with the total biomass quantified by crystal violet measured at 595 nm. Biofilm formation for each strain is shown.

a wide range of biofilm production, with 0.24 to 1.15 absorbance values.

Efficacy of linezolid on biofilm

The highest biofilm producing strains were selected for the following experiments, since we were interested in determining the inhibition of biofilm growth. To carry this out, the biofilm was exposed to LZD at subMIC over the whole growth period in order to evaluate the antibiotic effect. The clinical isolates MRSA 1, MRSA 2, the reference MRSA ATCC 4300 and the MSSA ATCC 29213 strains showed an inhibition percentage respect to the control (untreated sample) of 51.6%, 77.3%, 29.2% and 29.4%, respectively. In addition, the mature biofilm was treated with the antibiotic at 10× MIC and 100× MIC, and the biomass reduction was between 13% and 57% (ESI-Fig. 2†). These results may be attributed to the features of the mature biofilm matrix, since as previously described, the access of antibiotics into biofilms is delayed because it reduces the physical contact between antimicrobials and planktonic cells.[18,19] Moreover, the particular way each strain forms mature biofilms might have an impact on the antibiotic activity.[20] Related to this, Schilcher *et al.* (2016)[21] demonstrated that clindamycin has diverse effects on *S. aureus* biofilm formation at subinhibitory concentrations, which highly depend on the strain background. In a depth analysis on the model strain LAC wt (the mutant has a complete deletion of the *agr* locus) from the USA300 clonal lineage, they showed that subinhibitory concentrations of clindamycin alter the biofilm matrix composition by changing autolysis and eDNA release. These aspects produced an increasing of adhesion factors and secreted proteins which likely leads to an interaction between the matrix components, which might result in a more compact and stable biofilm. Since the increase in biofilm formation and alteration of the matrix composition might impact on *S. aureus* biofilm associated infections, so clindamycin should be dosed as high as possible in order to prevent subinhibitory clindamycin concentrations in affected tissues and biofilms formation.

To discriminate between living and dead cells another quantification method based on the metabolic activity, XTT, was used in the present study. The viability of cell growth in the biofilm and the mature biofilm exposure to LZD were measured for 24 hours employing the XTT assay. It was clear that each strain produced different levels of biofilm structure (Fig. 2).

Although all the strains produced a biofilm matrix, MRSA 1 formed the largest quantity and had a sustained growth after 24 h. In the case of MRSA 2, initially it displayed the same trend as MRSA 1 until 15 h, after which it presented a phase of reduced growth. Both reference strains exhibited a similar behavior of exponential growth, which entered a stationary phase after 12 h. It was evident that the clinical isolates increased their metabolic activity from earlier periods of biofilm production, whereas the ATCC strains displayed less activity in the early stages. In general, after 6 hours, a slow induction phase was observed in the reference strains, and the *S. aureus* strains showed no significant changes in biofilm growth between 12 and 24 h, with a similar pattern being observed for the reference strains.[22]

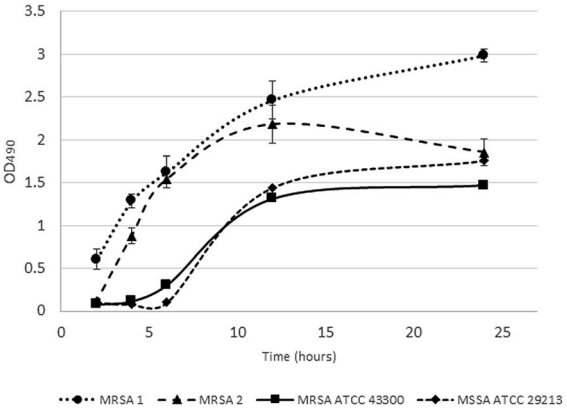

Fig. 2 Biofilm growth curves over time of *Staphylococcus aureus* clinical strains, MRSA 1 and MRSA 2, and reference MRSA ATCC 43300 and MSSA ATCC 29213 strains, evaluated by XTT assay at 490 nm. Bacterial viability was measured at 0, 4, 6, 12, and 24 hours. Each bullet point is derived from four duplicates of four independent experiments carried out on different days. Errors bars represent standard deviations of the means.

Also, it appeared that reference strains produced only moderate biofilms, whereas the two clinical isolates formed fully settled biofilms,[18] which was confirmed by using the CV and XTT, techniques to determine the biomass quantity.

Taking into account that, biofilm cells are organized into structured communities embedded within an extracellular matrix, activity within biofilms would be dependent on nutrient access and availability of oxygen, together with removal of waste products. These factors may vary because of inherent differences in the biofilms produced by the different microorganisms tested here, resulting in variations in biofilm metabolic activity.[18]

Interestingly, there were no significant viability differences for the tested strains when they were exposed to LZD at 10× MIC or 100× MIC (ESI-Fig. 3†). The high viability reduction obtained with the lower LZD concentration (10× MIC) encouraged us to investigate further the biofilm behavior after treatment with the

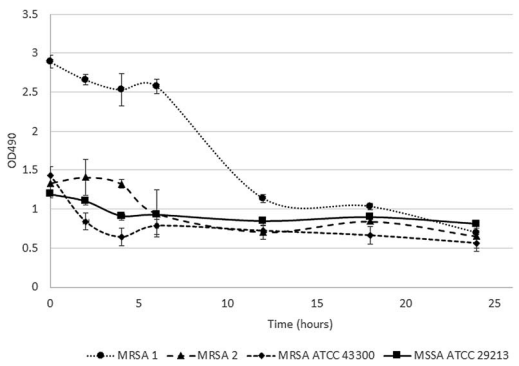

Fig. 3 Biofilm inhibition curve over time of *Staphylococcus aureus* clinical and reference strains by assessing biofilm viability with an XTT assay. The mature biofilms were exposed to LZD (10× MIC) at 0, 4, 6, 12 and 24 h. Each bullet point is derived from four duplicates of four independent experiments carried out on different days. Errors bars represent standard deviations of the means.

antibiotic. Related to this, once again, MRSA 1 presented the highest biofilm production (Fig. 3). Nevertheless, a notable reduction of 76% in the metabolic activity respect to the control was obtained with 20 μg mL^{-1} (10× MIC) of LZD after 24 h exposure, with this effect on activity after 12 h of treatment revealing a decreased of 61%. The reduction for MRSA 2 was 51%, and the strain followed a similar pattern to that of MRSA 1, with a 47% drop in the viability being observed after a 12 h exposure. In the case of the reference strains, MRSA ATCC 43300 exhibited a diminished activity of around 60.8%, and for MSSA ATCC 29213 this decrease was approximately 32% after 24 h.

Biofilm eradication

To try to reinforce these results, scanning electron microscopy (SEM) images of the untreated strain MRSA 1 (Fig. 4A) were obtained, and these revealed a moderately uniform thickness throughout the biofilm matrix, with the MRSA 1 biofilm presenting more bunched layers of cell clusters compared with the ATCC strain (Fig. 4E). Mature biofilms were exposed to LZD for 12, 18 and 24 h to study the time required to eradicate the biofilms. After 12 h of contact with LZD and MRSA 1, the images showed a reduced density of cell groups (Fig. 4B). Subsequently, the biofilm sample appeared to consist of only one assembled crowd (Fig. 4C), which after 24 h displayed a sharp drop in the number of cell clusters, with only a few independent isolated cells remaining stuck to the glass (Fig. 4D). By measuring the biofilm area on the cover glass (ESI-Table 1†), we were able to calculate the biofilm eradication percentages. These values were 34.7%, 98.2% and 99.8% after 12, 18 and 24 h of treatment, respectively.

Biofilm eradication was also confirmed by SEM for MRSA ATCC 43300, which at 12 h (Fig. 4F) was 83.8%. Then, between 12 and 18 hours (Fig. 4G) the farther decrease of cell density was not significant and attained 80.8% biofilm elimination. A few spherical coccus of MRSA ATCC 43300 were appreciated after exposure to LZD for 24 h (Fig. 4H), with LZD eradicating 98.6% of the sample on the cover glass. The substantial differences in biofilm production between the clinical and the reference strain can be observed in Fig. 4A and E. These results suggest that LZD can affect the structure of the biofilm and adhesion between the bacteria. Conversely, the biofilms grown in the presence of LZD were not well organized, as a result of low biofilms, the cell aggregation and cell-to-cell connections were prevented (as shown by SEM images), resulting in loosely arranged cell that can be easy disrupted.

The SEM tool provided useful information about the density of the bunch cell clusters and matrix (Fig. 5A and B), which was markedly higher in the clinical strain. Our SEM images revealed the ability of LZD eradicating biofilms of *S. aureus* at different times. It has been widely described that multiple factors influence biofilm formation in *S. aureus*.[23] According to Beenken *et al.* (2003),[24] the three principal regulatory genes, are *icaADBC*, the accessory gene regulator (*agr*) and the staphylococcal accessory regulator (*sarA*). Thus, our findings, which demonstrated biofilm heterogeneity, may be attributed to differences in gene regulation and expression.

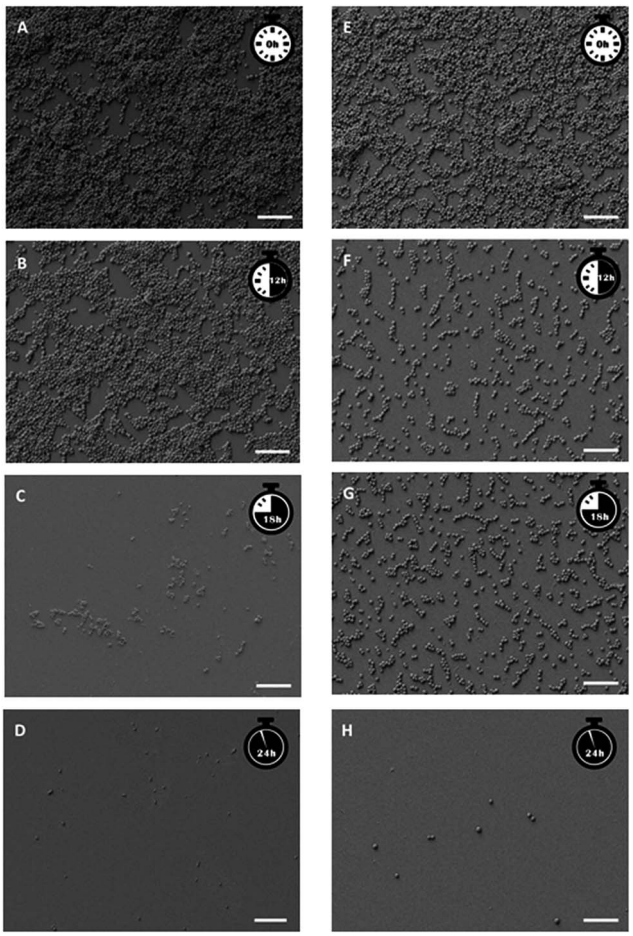

Fig. 4 Biofilm eradication over time caused by LZD. The MRSA 1 and MRSA ATCC 43300 images were taken by scanning electron microscopy. Untreated samples of MRSA 1 and MRSA ATCC 43300 displayed the biofilm structure (A and E, respectively). Biofilms treated with LZD (10× MIC) for 12 h (B and F), 18 h (C and G) and 24 h (D and H) of MRSA 1 (left column) and MRSA ATCC 43300 (right column) are shown. Scale bar = 10 μm.

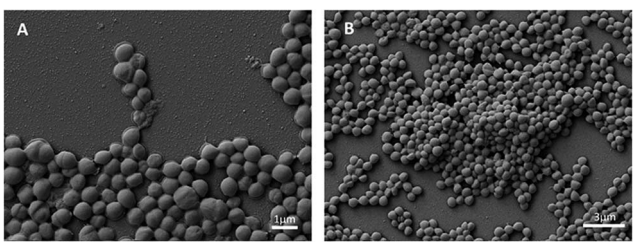

Fig. 5 Biofilm matrices of MRSA 1 and MRSA ATCC 43300 are displayed. SEM images of clinical (A) and reference (B) strains show the bunched cell cluster and the highest biofilm matrix produced in the clinical strain. Scale: white bar.

Other authors have studied LZD performance against diverse types of biofilm associated with, for instance, *N*-acetylcysteine[13] and antimicrobials such as daptomycin,[25] fosfomycin, levofloxacin[26] and rifampicin.[27–29] Baldoni *et al.* (2008)[27] reported a better enhancement performance between LZD and rifampicin killing planktonic bacteria and eliminating biofilms. In

the same report the association is also used for the treatment of implant-associated infections. The last study, the range of LZD concentrations employed were 1 to 4× MIC, whereas in our experimental conditions we achieved a 99.8% biofilms eradication at 10× MIC in MRSA 1.

Other studies performed *in vivo*, as the reported on MRSA experimental pneumonia in pigs, showed that LZD reached a better response than vancomycin, which was attributed to a better pharmacokinetic and pharmacodynamics profile of LZD.[30] Also, Soriano *et al.* (2007)[31] suggested in their study done with 85 patients that oral LZD is an effective alternative for treating orthopedic implant infections. Further, Fernandez-Barat *et al.* (2012)[32] investigated the systemic treatment with LZD in tracheal tubes in a model of MRSA pneumonia in pigs, demonstrating that this oxazolidinone limits biofilm development.

In summary, although, the specific mechanism by which LZD inhibits biofilms are still not entirely understood,[11] our results support the evidence that LZD is an efficient antimicrobial for treating MRSA biofilms.

Conclusions

Here we showed that the behavior of the clinical methicillin-resistant strains was different to the reference strains, and in some cases the former produced more biofilms. It is important to highlight the effect of LZD as a potent antimicrobial to eliminate biofilms. Although previous evidence and data suggested that most antistaphylococcal agents are not effective by themselves for eliminating mature biofilms, we demonstrated a useful antibiotic profile against methicillin-resistant strains. It is necessary to conduct experiments with clinical strains due to their different behaviors in order to gain better understanding of antibiotic impact on the bacterial physiology. In particular, we now intend to examine LZD efficacy against biofilms on medical devices.

Author's contribution

SRM and DMR carried out the experiments. SRM wrote the paper under the supervision of MCB and VA. All authors read and approved the final manuscript.

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